Cell volume-regulated human kinase h-sgk

The present invention relates to the cloning and characterization of a human serine/threonine kinase (h-sgk: serum and glucocorticoid dependent kinase). The invention furthermore relates to reagents for diagnosing conditions associated with a change in cell volume and/or in "macromolecular crowding" in the body, such as, for example, hypernatremia, hyponatremia, diabetes mellitus, renal failure, hypercatabolism, hepatic encephalopathy, inflammation and microbial or viral infections. The present invention additionally relates to pharmaceuticals comprising the h-sgk, nucleic acids which code for the h-sgk, or receptors, in particular antibodies, which specifically bind to the h-sgk.

Even when the extracellular osmolarity is constant, the constancy of the cell volume is continuously challenged due to transport across cell membranes and cellular metabolism, i.e. production and breakdown of osmotically active substances.

Cell swelling and shrinkage disturb the intracellular environment by diluting and concentrating, respectively, cellular macromolecules which lead to extensive impairment of cellular functions. This is why cells have developed a large number of cell volume-regulating mechanisms. Cell swelling leads, in most tissues, to cellular release of ions due to activation of ion channels and KCl cotransport. Cell shrinkage conversely leads to cellular uptake of ions due to activation of NaCl/KCl cotransporter and Na^+/H^+ exchanger.

Furthermore, cell shrinkage stimulates cellular accumulation and cell swelling stimulates cellular release of osmolytes, molecules which are specifically used to generate intracellular osmolarity [Burg, M.B., Am. J. Physiol. 268: F983-F996, 1995].

Finally, changes in the liver cell volume influence hepatocellular metabolism and gene expression [Häussinger et al. (1994) Am. J. Physiol. 267, E343-E355]. Cell swelling acts like an anabolic signal which stimulates protein and glycogen synthesis and inhibits protein and glycogen breakdown. Conversely, cell shrinkage acts as a catabolic signal by promoting the breakdown of glycogen and proteins and inhibiting the synthesis of proteins and glycogen [Häussinger et al. (1994) Am. J. Physiol. 267, E343-E355].

The cell volume has been recognized as a crucial element in the regulation of hepatocellular metabolism by hormones, cellular amino acid uptake and oxidative stress.

The signal mechanisms which couple cell function to the changes in the cellular hydration state are substantially unknown. Changes in the cell volume achieve their various effects on cell function partly by stimulating or suppressing the expression of particular genes, whose products then influence the expression or activity of a large number of cell components. In order to discover genes which are increasingly expressed on cell swelling, we carried out a differential mRNA

fingerprinting assay on cDNAs from hepatocytes which had been exposed either to isotonic or to anisotonic extracellular fluid. This resulted in a plurality of bands which showed differential expression rates on use of different primers.

It has been found, surprisingly, that the expression of one of these bands was stimulated under hypertonic conditions and inhibited under hypotonic conditions. The cDNA sequence of this band, whose expression is influenced in a particular way by changes in cell volume, has been analyzed in detail. It was found by sequence comparison that there is no similarity with any previously known human gene. The gene which has been found, whose nucleotide sequence is depicted in Fig. 1, surprisingly codes for a kinase, a putative serine/threonine kinase. Its sequence is depicted in Fig. 2 as well as in Fig. 1. It is highly homologous with previously known rat sgk (serum and glucocorticoid dependent kinase), a kinase whose expression is increased by serum and glucocorticoids. A dependence of the rat sgk on cell volume has not previously been described, however.

The present invention accordingly relates to a human cell volume-regulated kinase (h-sgk) and to processes for producing it by genetic manipulation.

Expression of the h-sgk is greatly dependent on the cell volume. Cell swelling inhibits expression of the h-sgk, whereas cell shrinkage stimulates expression of the h-sgk. Furthermore, expression of the h-sgk is inhibited

by urea. Urea impairs, like changes in cell volume, the stability and thus the function of cellular proteins and the packing density of the cellular macromolecules, called macromolecular crowding [Minton, A.P., Mol. Cell. Biochem. 55: 119-140, 1983]. h-sgk expression is therefore a measure of the cellular macromolecular crowding. Transcription of the h-sgk is not, in contrast to rat sgk, influenced either by corticoids or by fetal calf serum (FCS), however.

The h-sgk is expressed in a large number of human tissues such as liver, heart, pancreas, muscle, kidney, lung, placenta, lymphocytes and several structures in the brain (hippocampus, nucleus caudatus, corpus callosum, substantia nigra, nucleus subthalamicus and thalamus).

It has emerged that the h-sgk has a considerable diagnostic potential for many diseases in which changes in cell volume play a crucial pathophysiological part. Expression of the h-sgk can be demonstrated by detecting and/or quantifying the mRNA by using suitable probes, for example in a Northern blot or by in situ hybridization, and the h-sgk itself can be detected, for example, using suitable antibodies in a Western blot or by immunohistochemistry. Suitable probes and antibodies have already been successfully checked for utilizability.

The present invention therefore also relates to the diagnostic use of the h-sgk, its fragments or the relevant nucleic acids coding therefor. The diagnostic techniques

which can be used are known to the skilled worker. These may be all immunoassay formats known from the prior art, such as, for example, Western blot or enzyme linked immunosorbent assay (ELISA), but also homogeneous assay formats not bound to a solid phase. Conceivable examples are competitive assay variants, but also indirect assays or designs on the sandwich principle are also directly possible. It is likewise possible to employ the labeling techniques known to the skilled worker. All types of nucleic acid detection techniques can be used, such as, for example, Southern blot, Northern blot and all variants of the hybridization techniques, including in situ hybridization.

The h-sgk can be detected both in body fluids, for example, blood, plasma or serum, and in solid tissues, for example biopsy material. Detection of the h-sgk is indicated wherever changes in the cell volume or in the macromolecular crowding in the body occur, such as in hypernatremia, hyponatremia, diabetes mellitus, renal failure, hypercatabolism, hepatic encephalopathy, inflammation and infections.

Furthermore, dysfunction of the h-sgk might lead to impaired regulation of hepatic metabolism. Detection of the h-sgk would therefore be useful for diagnostic elucidation of fructose intolerance and hyper- and hypoglycemic states.

Hypernatremia: This is a life-threatening disturbance which occurs, for example, when there is osmotic diuresis and water diuresis due to central or

nephrogenic diabetes insipidus. Central diabetes insipidus results from a genetic defect, craniocerebral trauma, damage to hypothalamic neurons due to inflammations, hypoperfusion, tumors, consumption of alcohol, opiates and some drugs. Nephrogenic diabetes insipidus results from genetic defects, hypokalemia, hypercalcemia, protein deficiency, pyelonephritis, and treatment with various drugs etc. As is shown in experiments on cultivated liver and kidney cells, an increase in the extracellular Na^+ concentration, which is always associated with an increase in the extracellular osmolarity too, results in increased expression of the h-sgk. The kinase can thus be used as indicator of the extent of cell shrinkage and be employed for monitoring the therapy. Surveillance of this type is important inasmuch as fatal cell swelling may occur on occasion if the correction of hypernatremia is too rapid, despite extracellular hyperosmolarity.

Hyponatremia: Hyponatremia below 130 mmol/l is found in about 1-2% of all hospitalized patients. The causes of this life-threatening disturbance are diabetes mellitus, ketonuria, hepatic insufficiency, diuretics, opiates, various drugs, osmotic diuresis, bicarbonaturia, adrenal insufficiency, salt-loss nephritis, nephrotic syndrome, increased secretion of ADH and losses of isotonic fluid (for example diarrhea) with replacement only of water. If the hyponatremia is the result of an increase in other osmolytes in the blood, then the cell volume and expression of the h-sgk remain normal. However, if the hyponatremia reflects a hypoosmolarity with cell swelling, then there is a reduction in h-sgk expression.

Thus, measurement of the h-sgk provides information about the presence of cell swelling and allows a rational decision to be made about the therapeutic procedure. The kinase can be employed to check progress during therapy. Correction of hyponatremia which is too rapid may result in cell shrinkage, which is occasionally fatal.

Diabetes mellitus: Hyperglycemia occurs in diabetes mellitus and results in an increase in the extracellular osmolarity and thus causes cell shrinkage. The glucose undergoing glomerular filtration exceeds the maximum renal transport rate and, in this way, forces osmotic diuresis, in which Na^+ and water are lost. This may result in development of hyponatremia. The increased extracellular osmolarity and the oversupply of glucose promote the cellular production of sorbitol which, when the extracellular osmolarity falls, results in cell swelling. The cell shrinkage and cell swelling associated with diabetes mellitus are ascribed crucial importance in the pathophysiology [McManus et al., New England J. Med. 333: 1260-1266, Dermadash et al., Kidney intern. 50: 2032-2040, 1996]. Measurement of the h-sgk in a patient with diabetes mellitus permits the changes in cell volume to be estimated and thus provides a solid basis for compensating electrolyte disturbances. In this case too, observation of the progress can prevent excessive corrections.

Renal failure: In renal failure there is a massive increase in the urea concentration to levels which have a destabilizing effect on proteins, cause cells to

shrink and bring about a decrease in h-sgk expression. The destabilizing effect of urea is diminished by the formation of trimethylamines. When the changes in the urea concentration are rapid, the accumulation of trimethylamines cannot keep up, and disturbances of cellular metabolism are to be expected owing to the changes in cell volume. Determination of the h-sgk may reveal an imbalance between destabilizing urea and stabilizing trimethylamines. Therapeutic administration of trimethylamines would, where appropriate, be indicated if the h-sgk is greatly depressed.

Hypercatabolism: In a number of catabolic states, such as sepsis, burns, acute pancreatitis, major operations, changes in the volume of muscle cells correlating with the extent of hypercatabolism have been detected. Cell shrinkage in fact leads to enhancement, and cell swelling to inhibition, of proteolysis. Determination of the h-sgk might justify in the individual case the use of therapeutic measures suitable for counteracting cell shrinkage, such as administration of glutamine [Häussinger et al., Lancet, 341: 1330-1332, 1993] or of osmolytes [Burg, M.B., J. Exp. Zool. 268(2): 171-5, 1994].

Hepatic encephalopathy: There is compelling evidence that hepatic encephalopathy is brought about by swelling of glial cells [Norenberg, M.D., Exp. Neurol. 53(3): 213-220, 1994]. It is in fact possible to detect a decrease in the osmolyte inositol in the brain in cases of liver disease [Kreis et al., NMR Biomed. 4: 109-116, 1991]. Complete disappearance coincides with the onset

of encephalopathy. Development and use of suitable substrates for the h-sgk might allow the h-sgk activity in the brain to be measured and counter-regulated even before the encephalopathy occurs. Where appropriate, h-sgk expression in more readily accessible tissues might also be used as indicator of volume changes in glial cells.

Alzheimer disease: Recent evidence points to increase of peripheral cell volume in Alzheimer disease. Moreover, the osmolyte inositol is enhanced in patients with Alzheimer disease, but not in dementia of other causes. H-sgk expression may contribute to diagnosis of Alzheimer disease.

Infections/Inflammation: Sepsis is associated with extensive cell shrinkage [Häussinger et al., Lancet 1993, 341: 1330-1332] with the corresponding occurrence of hypercatabolism. In fact, the cell volume plays an important part in the pathogen-host relationship. Expression of the h-sgk might be a valuable parameter for assessing the pathophysiology of infections. In situ hybridisation reveals marked increase of tissue levels of h-sgk in inflammatory diseases, such as hepatitis, pancreatitis, Morbus Crohn, or glomerulonephritis. Moreover, h-sgk expression is enhanced by TGFβ which has been implicated in progressive fibrosis such as liver cirrhosis, lung fibrosis and progressive renal failure. H-sgk expression has indeed been found enhanced in patients with chronic renal failure.

Hyperglycemia/hypoglycemia/lactacidosis: Diminished or enhanced expression and/or function of the h-sgk might result in disturbances of carbohydrate metabolism as observed in association with cell shrinkage and cell swelling [Lang et al., Pflügers Arch. 413: 209-216, 1989]. A diminished function would result in the threat of hypoglycemia. Enhanced function might be followed, on the one hand, by hyperglycemia or, on the other hand, by lactacidosis. Thus, in the diagnostic elucidation of hyperglycemia, hypoglycemia and lactacidoses of unclear origin, it would always also be expedient to investigate the expression and function of the h-sgk.

The present invention is additionally explained further by the following detailed description and, furthermore, described by the examples and the claims.

Detailed description of the invention

Materials and methods

Materials: Fetal calf serum and DMEM (Dulbecco's modified Eagle's medium) were purchased from GIBCO/BRL (Eggenstein, Germany), enzymes from STRATAGENE (Heidelberg, Germany) and BOEHRINGER MANNHEIM (Mannheim, Germany), α-[^{35}S]-dATP from ICN (Eschwege, Germany), SuperScript® reverse transcriptase from GIBCO/BRL. PCR® (polymerase chain reactions) were carried out in a Crocodile® II thermocycler (APPLIGENE ONCOR, Heidelberg, Germany) using Prime Zyme® DNA polymerase and PCR buffer from BIOMETRA (Göttingen, Germany). RAP-PCR primers were purchased from STRATAGENE, and sequencing primers from

MWG (Ebersberg, Germany). Manual sequencing was carried out on an S2 sequencer from GIBCO/BRL using the Fidelity® DNA sequencing system (APPLIGENE ONCOR).

Cell culture: HepG2 human hepatoma cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) with 5% CO_2, 5 mM glucose, pH 7.4, which was supplemented with 10% (vol/vol) fetal calf serum (FCS) at 37°C. Before the RNA isolation, the cells were cultivated to 90% confluence and kept in basal medium Eagle's (BME, GIBCO/BRL) without fetal calf serum for 12 hours. The extracellular osmolarity was varied by adding or removing defined amounts of sodium chloride without changing the other components of the BME medium. In experiments to test the effects of amino acids, the cells were kept in an extracellular solution free of amino acids for two hours before adding the amino acids.

RAP-PCR: RNA fingerprinting PCR (RAP-PCR) was carried out as described previously [McClelland et al., 1994, Nucleic Acids Res. 22, 4419-4431]. After electrophoresis through a 4% acrylamide/7 M urea polyacrylamide gel, the PCR products were visualized by silver staining [Sanguinetti et al., Biotechniques 17, 914-921, 1994]. All the bands which were visible only under one condition (hypertonic or hypotonic) were subsequently confirmed by reverse transcription and PCR with RNA from new cultures. The RAP-PCR was carried out with four different primer pairs for the cDNA synthesis and PCR amplification. In addition, different temperatures between 30°C and 40°C were chosen in the first

amplification round. Together with these modifications, a total of 64 PCR runs were carried out.

Isolation of the bands: Bands which showed reproducible differences were cut out under sterile conditions. The amplicon was eluted in 100 µl of buffer (50 mM KCl, 10 mM TRIS-Cl pH 9.0, 0.1% Triton X 100) at 70°C overnight. Reamplification by PCR was carried out with 3.0 µl of eluate using suitable primers (250 nM), 200 µM dNTP, 1x low-salt buffer (STRATAGENE) with 1.5 mM $MgCl_2$ and 5 units of Taq+® DNA polymerase (STRATAGENE) with the following temperature cycle profiles: one cycle at 95°C for 60 sec, 30 cycles at 95°C (15 sec), 55°C (15 sec), 72°C (60 sec) and finally at 72°C for 5 minutes. After confirmation by PAGE that only one defined amplicon having the expected length had been produced, this amplicon was used directly for forming the probe.

Northern analysis: Digoxigenin (DIG)-coupled probes were produced by direct PCR labeling of the various amplicons using the suitable primers and the conditions as described above apart from the fact that the following dNTP concentrations were used: 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 190 µM dTTP and 10 µM DIG-dUTP (BOEHRINGER). Northern blots were prepared using 20 µg of total RNA or 2 µg of poly(A)-RNA, which had been separated by electrophoresis through 1% agarose gels in the presence of 2.2 M formaldehyde. Equivalent loading of probes in the investigation of poly(A)-RNA was checked by ethidium bromide staining of ribosomal RNA

bands or by DIG-labeled antisense RNA probe using the human heterogeneous nuclear ribonucleoprotein C1 as internal standard. The size of the RNA was estimated using the DIG-labeled molecular weight marker I (BOEHRINGER). Vacuum blotting (APPLIGENE ONCOR Trans DNA Express Vacuum Blotter) was used for transfer to positively charged nylon membranes (BOEHRINGER) which were then crosslinked by ultraviolet light (STRATAGENE UV Stratalinker® 2400). Hybridization was carried out in DIG-Easy-Hyb® (BOEHRINGER) with a probe concentration of 25 ng/ml or 100 ng/ml at 50°C or 65°C for DNA probes or RNA probes overnight. Probes which showed different expression were subcloned using the pCR-Script SK(+) cloning kit (STRATAGENE) and checked in Northern blots. The Northern blots which are shown hereinafter were derived from these subclones.

Other methods: DNA sequencing of the pCR clone was carried out with the Fidelity® DNA sequencing system (APPLIGENE ONCOR). Sequencing products were labeled with α-[^{35}S]-dATP and fractionated on a 6% polyacrylamide/8 M urea sequencing gel. The GenBank data were scanned for homologous sequences using the FASTA computer program [Pearson, W.R. & Lipman, D.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2444-2448]. The sequence of the complete h-sgk cDNA was obtained with the aid of the I.M.A.G.E. consortium clone ID 42669 from the TIGR/ATCC special collection of human cDNA clones. The gene data were examined with the aid of the European Molecular Biology Laboratory EMBL (Heidelberg), the BLAST network service

and - for the protein alignments - the BLITZ server on the newest edition of the SwissProt protein database.

Nucleotide sequence accession number: The h-sgk cDNA sequence was entered into the GenBank database under accession number Y10032 and was inaccessible until March 1, 1997.

Results

Differential gene expression in HepG2 cells in hypotonic, isotonic and hypertonic extracellular fluid: mRNA was isolated from HepG2 cells which had been pretreated for 1 or 2 hours with hypotonic (hypotonic I: minus 100 mosmol/l due to removal of 50 mM NaCl and hypotonic II: minus 50 mosmol/l due to removal of 25 mM NaCl compared with isotonic control medium), isotonic (with total osmolarity of 290 mosmol/l and an NaCl concentration of 114 mM) or hypertonic (plus 50 mosmol/l by addition of 50 mM raffinose) medium. The mRNA was used as template for the RAP-PCR with arbitrary primers. The products of the RAP-PCR were loaded onto denatured polyacrylamide gels and fractionated in parallel lanes for comparison. Several bands showed differential expression on use of several primers. Four differential bands from the RAP-PCR gels were analyzed further: two proved in the subsequent Northern blot analysis to be false-positive, one band was enhanced by hypotonic and hypertonic conditions but its sequence showed no similarity with any previously known cloned gene. One band of about 500 base pairs showed increasing expression with increasing extracellular osmolarity (hypotonic I-

hypotonic II-isotonic-hypertonic). This band was purified from the gel and reamplified using the primer RAP-A4. After PCR labeling with digoxigenin, Northern blots were made using this amplicon in order to confirm differential expression in various cell preparations which had been pretreated for two hours with hypotonic I, isotonic and hypertonic medium.

A single transcript of about 2.6 kilobases was greatly influenced by the changes in the extracellular osmolarity (Fig. 1). The amount of transcript was reduced when the osmolarity decreased and was enhanced when the osmolarity increased.

Cloning and sequencing of the differentially regulated h-sgk gene. The PCR product with a length of 500 base pairs was subcloned into the PCR II vector, and a new probe was produced with this construct in order to demonstrate identity between the original and the sub-cloned DNA fragment. Rehybridization of a Northern blot using this probe led to identical results as with the original probe. In addition, a Southern blot analysis was carried out with the new construct and hybridized with the original construct. Strong hybridization after two high-stringency washes confirmed the identity of the sequence.

Sequence analyses in both directions showed the presence of the primers used on both sides of the amplicon. An amino acid sequence translated by one reading frame of the nucleotide sequence showed 95% identity with the carboxyl-terminal amino acid sequence of the rat sgk

(serum and glucocorticoid regulated protein kinase), a new member of the serine/threonine protein kinase protein family which was cloned from a rat mammary gland tumor cell line [Webster et al., Mol. Cell. Biol. 13, 2031-2040, 1993a]. Because of the great similarity, the name h-sgk (human) was chosen for the new protein.

The Genbank database was scanned for similar human sequences using the FASTA computer program. Several EST (Expressed Sequence Tags) DNA sequences from the TIGR/ATCC special collection of human cDNA clones showed 100% sequence agreement with parts of the h-sgk cDNA fragments. After multiple alignments of 30 different TIGR/ATCC human cDNA clones with the rat sgk cDNA sequence (Genbank accession number L01624) and with the h-sgk DNA fragment, it was assumed that the I.M.A.G.E. consortium construct with the clone ID 42669 from a human infantile brain library has the complete coding sequence of the h-sgk. Sequence analysis of this construct with coinciding sequences in the sense and antisense directions revealed a cDNA sequence of about 2.4 kilobases. In order to demonstrate involvement of the complete h-sgk, the 5' end of the clone (nucleotides 1-285 of the coding sequence) was subcloned into the pCR II vector and hence a new probe was produced with this construct. Hybridization of a Northern blot with this probe resulted in identical results as with the original probes (Fig. 1). The longest reading frame in the clone investigated (1.3 kb) afforded a 431 amino acid protein with an overall identity of 98% with the rat sgk protein.

Regulation of h-sgk expression by changing the extracellular osmolarity: In order to investigate the effect of changes in the extracellular osmolarity on the amounts of h-sgk transcript, HepG2 cells were incubated for various times in hypotonic (190 mol/l), isotonic (290 mosmol/l) and hypertonic (390 mosmol/l) BME medium without FCS. The h-sgk mRNA concentrations increased greatly within 60 min in hypertonic solution. The initial rise was evident within 30 minutes and reached a maximum within two hours. Induction of the h-sgk therefore directly follows the change in the osmolarity. The transcript concentrations increased further over 4 to 8 hours in hypertonic extracellular BME medium, and then gradually fell again over the course of the next 16 to 24 hours to the initial concentrations. On the other hand, the h-sgk transcript concentrations decreased rapidly in hypotonic extracellular solution, the decrease being evident after only 30 minutes and reaching a maximum within two hours.

Different osmolarities (140, 190, 240, 290, 340, 390 and 440 mosmol/l) showed differences in the expression of the h-sgk within two hours. A steep correlation of h-sgk expression with the extracellular osmolarity was detectible over the entire range. A 30% increase in osmolarity was associated with an approximately tripling of kinase expression. An increase in osmolarity from 290 to 340 mosmol/l, and a decrease in concentration from 290 to 240 mosmol/l, induced significant changes in h-sgk expression. The transcriptional control mechanism thus evidently reacts very sensitively to changes in osmolarity. Induction of h-sgk RNA was independent of

de novo protein synthesis. The increase in the transcript concentration in hypertonic BME medium is greater in the presence of the protein synthesis inhibitor cycloheximide (10 µg/ml) than in the absence of the inhibitor.

The rapid decrease in the h-sgk transcript concentrations immediately after reducing the extracellular osmolarity suggests that the h-sgk mRNA has a short half-life. In order to investigate the rate of decrease of the h-sgk transcript concentrations, HepG2 cells were treated with hypertonic medium (390 mosmol/l) for two hours in order to obtain a maximum increase in the h-sgk transcript concentrations. Then some of the cells were exposed to the RNA polymerase inhibitor actinomycin D (5 µg/ml), and the remaining cells to hypotonic medium (190 mosmol/l). After various times, RNA was prepared and the transcript concentrations of the two groups of cells were compared. Actinomycin D treatment resulted in a rapid decrease in the h-sgk transcript concentrations with an estimated half-life of about 30 minutes. Treatment of the cells with hypotonic extracellular medium resulted in an equally rapid decrease in the transcript concentrations.

Regulation of h-sgk transcript concentrations by isotonic changes in cell volume. In order to be able to distinguish between the effects of changes in the cell volume, the ionic strength and the osmolarity, the cell volume was manipulated by two different methods while keeping the ionic strength and osmolarity the same.

Within two hours of isotonic cell shrinkage due to inhibition of the NaCl/KCl cotransporter and of the Na^+/H^+ exchanger with bumetanide and 3-methylsulfonyl-4-(1-piperidino)benzoylguanidine (EP-0 416 499) there was an increase in h-sgk expression, an effect which was further enhanced by additionally increasing the extracellular osmolarity. Cell swelling by offering various amino acids (amino acid mixture of 1x BME amino acids, GIBCO/BRL) conversely resulted within two hours in a decrease in h-sgk transcript concentrations. It is thus the cell volume, not the osmolarity or the ionic strength, which regulates h-sgk expression.

In order to check whether expression of h-sgk in HepG2 cells is, similar to the rat sgk in mammary gland tumor cells [Webster, M.K. (1993) Mol. Cell. Biol. 13, 2031-2040], regulated by glucocorticoids or fetal calf serum (FCS), HepG2 cells were incubated with dexamethasone (1 µM) or with FCS (10%) for two to 12 hours. No effect of glucocorticoids or FCS on the h-sgk transcript concentrations in HepG2 cells could be found in Northern blots.

Regulation of sgk transcript concentrations by the extracellular osmolarity in Madin Darby canine kidney (MDCK) cells. In order to check whether the observed dependence of h-sgk expression on cell volume is a peculiarity of HepG2 cells, the canine kidney epithelial cells MDCK were exposed to hypotonic (190 mosmol/l) and hypertonic (390 mosmol/l) BME medium for two hours. It was possible to detect h-sgk transcripts with a

length of about 2.6 kilobases even after several high-stringency washing steps with 0.5 x SSC (standard saline citrate) at 65°C, indicating great homology of the sgk gene sequences between different species.

Changes in the extracellular osmolarity had a similar effect on the transcript concentrations in MDCK cells as in HepG2 cells.

Tissue-specific expression of h-sgk. A premade Multiple Tissue Northern Blot (CLONTECH, Heidelberg, Germany) was investigated with the h-sgk DNA probe. Expression of h-sgk shows a certain tissue specificity, with greatest expression in pancreas, liver and myocardium. Expression is somewhat less in placenta, lung and skeletal muscle. There is low but detectable expression in brain and kidney. In human brain tissue, expression is greatest in the substantia nigra and the corpus callosum, average in the corpora amygdala, hippocampus, nucleus caudatus and nucleus subthalamicus, and least in the thalamus. It is of interest that a second transcript of 7 kilobases was found in almost all tissues, with greatest expression in the pancreas. This transcript is possibly another h-sgk mRNA due to alternative splicing or a gene homologous to h-sgk. The 7 kilobase transcript had not been found in HepG2 Northern blots.

Regulation of h-sgk expression by urea. The presence of urea in the extracellular space depresses h-sgk expression. The diminution in h-sgk expression was moderate at 50 mmol/l urea and extensive with 100 mmol/l urea.

Discussion

The human gene h-sgk, whose transcription is regulated by changes in the cell volume, codes for a putative serine/ threonine protein kinase with great homology with the sequence of rat sgk, which has recently been described as serum- and glucocorticoid-regulated gene from rat mammary gland tumor cells, as lesion-induced gene after CNS lesions in the rat brain [Imaizumi et al., Mol. Brain Res. 26, 189-196, 1994] and as gene induced by testosterone and follicle stimulating hormone in granulosa cells of the rat ovary [Richards et al., Recent Prog. Horm. Res. 50, 223-254, 1995]. The 49 kD h-sgk protein shows approximately 98% homology with the rat sgk protein with substantially conservative amino acid exchanges. It exhibits about 50% homology in its catalytic domain with several kinases of the second messenger family, such as rac protein kinase, protein kinase C, ribosomal protein S6 kinase, and cAMP-dependent protein kinase [Webster et al., (1993b) J. Biol. Chem. 268, 11482-11485, Webster et al. (1993a) Mol. Cell. Biol. 13, 2031-2040].

The level of expression of the 2.6 kilobase h-sgk transcript in HepG2 cells is greatly influenced by changes in the extracellular osmolarity. Increased transcript concentrations were found within 30 minutes after the extracellular osmolarity was increased. This induction was independent of de novo protein synthesis. The transcript concentrations fall within 30 minutes after the extracellular osmolarity is reduced. The decrease was as

rapid as the fall after inhibition of transcription by actinomycin D.

Changes in cell volume accordingly influence the h-sgk transcription rate. The reduced h-sgk transcription rate after osmotic cell swelling and the short half-life ensure rapid and efficient relation of h-sgk RNA transcript concentrations in HepG2 cells.

Isosmotic changes in the cell volume influence h-sgk expression in the same way. Cell shrinkage was achieved by inhibiting the essential ion transport mechanisms of the Na^+/H^+ exchanger and NaCl/KCl cotransporter by their specific blockers 3-methylsulfonyl-4-(1-piperidino)-benzoylguanidine (EP-0 416 499) and bumetanide. Cell swelling was brought about by adding amino acids and the subsequent hepatocellular accumulation of the amino acids via Na^+-dependent amino acid transporters such as, for example, system A, N, and ASC. The transcript concentrations correlated with the cell volume, not with the osmolarity.

After long-lasting osmotic cell shrinkage, the transcript concentrations increased steeply within the first half hour and then remained elevated for 8 hours before they gradually declined again. This long-lasting increase is apparently contradictory to the rapidity of cell volume regulation. Liver cells which have been shrunk or swollen osmotically do not, however, regulate their cell volume completely but still remain moderately shrunken or swollen after the rapid phase of volume

regulation [Häussinger et al., (1994) Am. J. Physiol. 267, E343-E355].

The remaining changes in cell volume might be responsible for the altered h-sgk expression.

Besides the cell volume itself, urea has a great effect on h-sgk expression. Urea exerts a destabilizing action on proteins and, in this way, imitates the effect of cell swelling. The h-sgk would thus be a sensor of protein stability or of the packing density of the cellular macromolecules. The destabilizing effect of urea is diminished by the formation of trimethylamines, whereby the destabilizing effect of urea is probably diminished in cases of renal failure.

The cellular effects of h-sgk are still uncertain. In particular, it is not yet possible at present to state with certainty whether the effects of h-sgk are involved in cell volume regulation. However, the effect of h-sgk is immaterial for use of h-sgk as diagnostic aid.

Despite the evident homology with the rat sgk sequence, we were unable to find any parallels to the regulation of the rat sgk. Neither serum (FCS) nor glucocorticoids (dexamethasone), both of which had showed a strong effect on sgk transcription in rat mammary gland tumor cells, affected h-sgk expression in HepG2 cells. It therefore appears that different h-sgk promoter sequences regulate expression of the protein in the various types of cells. Thus it is conceivable that h-sgk expression is not regulated exclusively by the

cell volume or the packing density. We were also able to detect a dependence of h-sgk expression on cell volume in kidney epithelial cells (MDCK) and in macrophages. The dependence of h-sgk expression on cell volume is thus not peculiar to HepG2 cells. The h-sgk 5'-flanking sequences in the various cells might uncover the regulating elements responsible for differences in sgk transcript expression. Like the previously described glucocorticoid- and serum-induced expression of sgk in the rat, the cell volume-induced expression of h-sgk RNA takes only 30 minutes. The half-life of 30 minutes for the h-sgk transcripts in HepG2 cells is just as short as the sgk half-life in rat mammary gland tumor cells, as shown by the experiments with the RNA polymerase inhibitor actinomycin D.

The h-sgk transcript is expressed in all human tissues investigated to date. Expression is particularly great in pancreas and liver, possibly because of the specialized epithelial function of these tissues.

Protein phosphorylation is a rapid and reversible mechanism for converting signals from the extracellular space into alterations of a large number of cell functions. The h-sgk protein kinase might induce, by phosphorylating specific proteins, some of the mechanisms regulating cell volume and represent a previously unknown link between cellular hydration and cell function.

Examples:

1. Procedure for Northern hybridizations:

10-20 mg of complete RNA or 1-2 mg of poly(A)-RNA were fractionated by electrophoresis in a 1% agarose gel in the presence of 2.2 M formaldehyde. Transfer to a positively charged nylon membrane took place with the aid of a Vacuum Blotter with 10 × SSC as transfer buffer for a period of two hours. Subsequently, the RNA was cross-linked covalently to the membrane by controlled-power UV irradiation. Hybridization of the specific probe (25 ng/ml) was carried out at 50°C overnight in a buffer specially developed for the purpose of non-radioactive hybridization (DIG Easy Hyb, BOEHRINGER). The probe used in this case was amplified by means of the polymerase chain reaction from the 3' end of the coding sequence of the relevant h-sgk (nucleotide 980-1480) and simultaneously labeled by including DIG-dUTP in the reaction buffer. After the blots had been washed twice in 2 × SSC at room temperature and in 0.5 × SSC at 65°C, the labeled probe was detected by an ELISA using an anti-digoxigenin antibody coupled to alkaline phosphatase which produced a chemoluminescence reaction in CDP-Star (BOEHRINGER) which was detected by autoradiography (average exposure time about two minutes).

2. Western blot analysis:

Details of antibody production: The rabbits were immunized by using two peptides from the h-sgk amino acid sequence: Pos.386-Pos.404 (DPEFTEEPVPNSIGKSPDS), Pos.416-Pos.431 (EAFLGFSYAPPTDSFL). The two peptides

were conjugated to KLH and to MAP, respectively, as carrier and injected intracutaneously with complete and incomplete, respectively, Freund's adjuvant. The injection and blood-sampling protocol followed standard procedures. The immune sera were purified by affinity chromatography, and the antibody fractions were collected and used at a concentration of about 1 mg/ml.

Immunoblot analysis: About 60 mg of total cellular protein were fractionated by electrophoresis through an SDS/7.5% polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were blocked in 3% BSA/5% milk powder/0.06% Tween 20 in PBS overnight. Primary (affinity-purified anti-h-sgk) and secondary (horseradish peroxidase-conjugated goat anti-rabbit IgG, Bio-Rad) were each incubated in 3% BSA/0.06% Tween in PBS at room temperature for one hour. An enhanced chemoluminescence kit (ECL, Amersham) was used for immunodetection.

3. In situ-hybridization:

15 mm frozen sections underwent counterfixation in 4% formaldehyde for 20 minutes, followed by two washing steps in 100 mM phosphate buffer pH 7.2 for 5 minutes each time. Proteinase K treatment (1 mg/100 ml) was followed by incubation in 0.1 M triethanolamine/0.225% acetic acid for 10 minutes. After renewed washing with 100 mM phosphate buffer, the sections were dehydrated in an ascending alcohol series. Prehybridization took place in hybridization buffer at 50°C, and the hybridization was carried out overnight. The probe used corresponds to the probe described for the Northern blots. Detection took place by enzymatic cleavage of an X-phosphate solution catalyzed by an alkaline phosphatase coupled to anti-digoxigenin antibodies.